EFFECTIVENESS OF VAPOROUS HYDROGEN PEROXIDE FOR THE DECONTAMINATION OF REPRESENTATIVE MILITARY MATERIALS

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ABSTRACT

The intentional release of biological agents has brought to the forefront the necessity of developing effective environmentally benign methods of decontamination for both the war fighter and the civilian population. While many chemical decontamination methods, including aqueous hydrogen peroxide, have been used or are under development for direct application, few vaporous methods are being evaluated for decontamination efficacy (McDonnell, G. *et al.*, 2002). Vaporous methods include, but are not limited to, formaldehyde, ethylene oxide and vaporous hydrogen peroxide (VHP®). While these gases are effective, the former two are toxic, carcinogenic and potentially explosive whereas the VHP process requires no neutralization prior to environmental release due to its rapid decomposition into two environmentally benign products: oxygen and water vapor. The VHP process therefore, is a viable alternative decontamination technology. As such, the Department of Defense (DOD) is interested in acquiring/developing a decontamination strategy to be used for militarily relevant surfaces(Heckert, R. *et al.*, 1997).

1. INTRODUCTION

In this study, we investigated the efficacy of VHP systems designed by the STERIS Corp. and the Edgewood Chemical Biological Center (ECBC), to provide a 6-log reduction from populations comprised of different microbiological agents and their surrogates. A collection of 10 test materials differing in their surface characteristics and composition, were contaminated with either spores or vegetative Gram-negative cells (Table 1).

In addition, experiments are being conducted in chambers of two sizes as well as the wing of a building to determine the sensitivity of the treatment to the effects of scale.

The intentional release of toxic agents is not limited to biological material but can extend to chemical agents and toxins. The VHP process has been demonstrated to be effective against some chemical warfare agents, and, through the addition of adjuvants exhibits enhanced effectiveness.

Current studies of the VHP process through the addition of an adjuvant, the use of test chambers of differing sizes, and the incorporation of militarily significant materials, suggest that the VHP process can be considered a broad-spectrum environmentally friendly and versatile decontamination method.

2. MATERIALS & METHODS

1. BACTERIAL SPORE PREPARATION

Bacillus spore stocks were prepared as described by Dang, *et. al*, with modification (Dang, J. L. *et al.*, 2001). Bacterial spores were harvested from 7-10 day –old cultures plated upon Lemko Agar. The spores were washed thrice in sterile distilled water (dH_2O), and collected by centrifugation for 15 min at 1965 X *g* etween washings. Spores were incubated in 70% (v/v) ethanol for 1 hr, collected by centrifugation as above, and subsequently incubated in dH_2O at 73°C for 1 hr. Spore stocks were titered and stored at 4°C.

2. PREPARATION AND TESTING OF GRAM NEGATIVE SURROGATE

Inocula of log-phase *Y. ruckeri* were prepared immediately before application to sterile coupons. Cells were grown in nutrient broth to an optical density at 420 nm of 0.9. Cells were collected by centrifugation for 10 min at 18,000 X *g* and suspended in 12 ml Peptone Water supplemented with 5% (v/v) fetal bovine serum, yielding $1E^9$ CFU/mL. 10 uL of cell suspension was

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Table 1. Bacterial strains and test materials

Bacterial Strain	Coupon Type
Bacillus anthracis NNR1Δ1	Aluminum
B. atrophaeus ATCC 49337	Air Force Top Coat
B. subtilis ATCC 19659	Butyl-coated Cloth
B. thuringiensis ATCC 35646	Chemical Agent Resistant Coating (CARC)
Geobacillus stearothermophilus ATCC 7953	Flott Glass
Yersinia ruckeri ATCC 29473	Galvanized aluminum
	Polyimid (Kapton)
	Nylon Webbing
	Runway Concrete
	Stainless Steel

distributed onto the surface of test coupons and dried in a laminar flow hood. Immediately upon drying, 1 set of coupons, in triplicate, was placed in a sterile saturated humidity chamber, while a second set of coupons underwent exposure to the VHP antimicrobial. Surviving cells were enumerated, as described below.

3. THE VHP ANTIMICROBIAL EXPOSURES AND ENUMERATION

Ten materials of varying surface characteristics were selected for the testing of the VHP antimicrobial (Table 1). $1E^7$ bacterial spores were deposited onto the surface of the test coupons as a 10 μL volume. The spore suspension was air-dried for a 1 – 18 hr prior to exposure to the VHP antimicrobial. Either of three test chambers, differing in volume was used to treat the contaminated test coupons. Bench-scale testing employed a 1.3 ft^3 chamber (Fig. 2) while larger test venues were $1E10^3$ ft^3 and $5E10^6$ in volume.

The time course of exposure can be divided into four components: dehumidification, conditioning, decontamination, and aeration. Dehumidification of the test chamber to a humidity level below 40% was achieved through the use of stand-alone dehumidifiers (Topaz). When the air was sufficiently dry, the VHP antimicrobial was introduced into the test chamber during the conditioning phase at such a rate as to yield a steady state concentration of 250 – 350 ppm prior to the beginning of the decontamination phase. Contaminated coupons were distributed into the test chamber for indicated periods of time. The addition of a gaseous adjuvant to the vapor phase VHP was achieved through use of a mass flow controller (Cole-Parmer). The concentration of ammonia in the final gaseous mixture was 8 ppm. At the conclusion of the exposure period, the decontaminant was catalytically degraded to oxygen and water (Jahnke, M. *et al.*, 1997), constituting the aeration phase.

Viability was determined by collection of the contaminant in 5 ml of buffered peptone H_2O, to which catalase had been added to a final concentration of 0.01% (v/v) followed by sonication, the addition of 10 ul of 1% Antifoam 289 (Sigma Aldrich Chemical Co.) and vortexing.

Viable cells were enumerated through serial dilution and subsequent growth on rich solidified medium. All experiments and all enumerations were performed in triplicate. Cell counts were performed using a Q-Count Colony Counter (Spiral Biotech). The enumeration of vegetative cells required slight modifications to the protocol, where sonication and vortexing times were reduced to 30 sec and 10 sec, respectively.

4. USE OF BIOLOGICAL INDICATORS

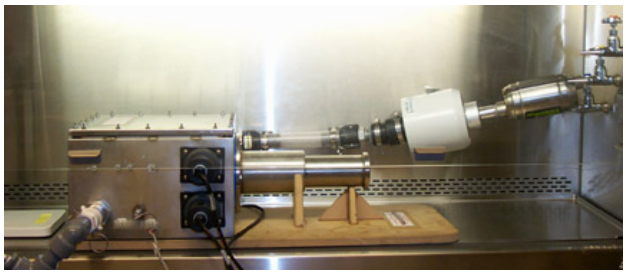

Fig. 1, Bench-scale test chamber

Biological indicators, purchased from several vendors, were exposed to the VHP antimicrobial and functioned as a confirmatory test for bacteriocidal effectiveness. The bacterial species used were *B. atrophaeus* (Raven) and *G. stearothermophilus* (Apex, Steris, Raven). Biological indicators were distributed throughout the venue in replicate and exposed to the VHP antimicrobial while either sheathed in a Tyvek® pouch or unsheathed (Fig. 2). Unsheathed coupons were suspended from sterile hooks, away from any potentially contaminating surfaces. Following exposure, coupons bearing the test spores were placed in a 5 ml volume of sterile growth medium and tested for viability. Cultures were incubated at the appropriate temperature for the species for a period of 7 days. Lack of turbidity following incubation was considered a non-viable sample.

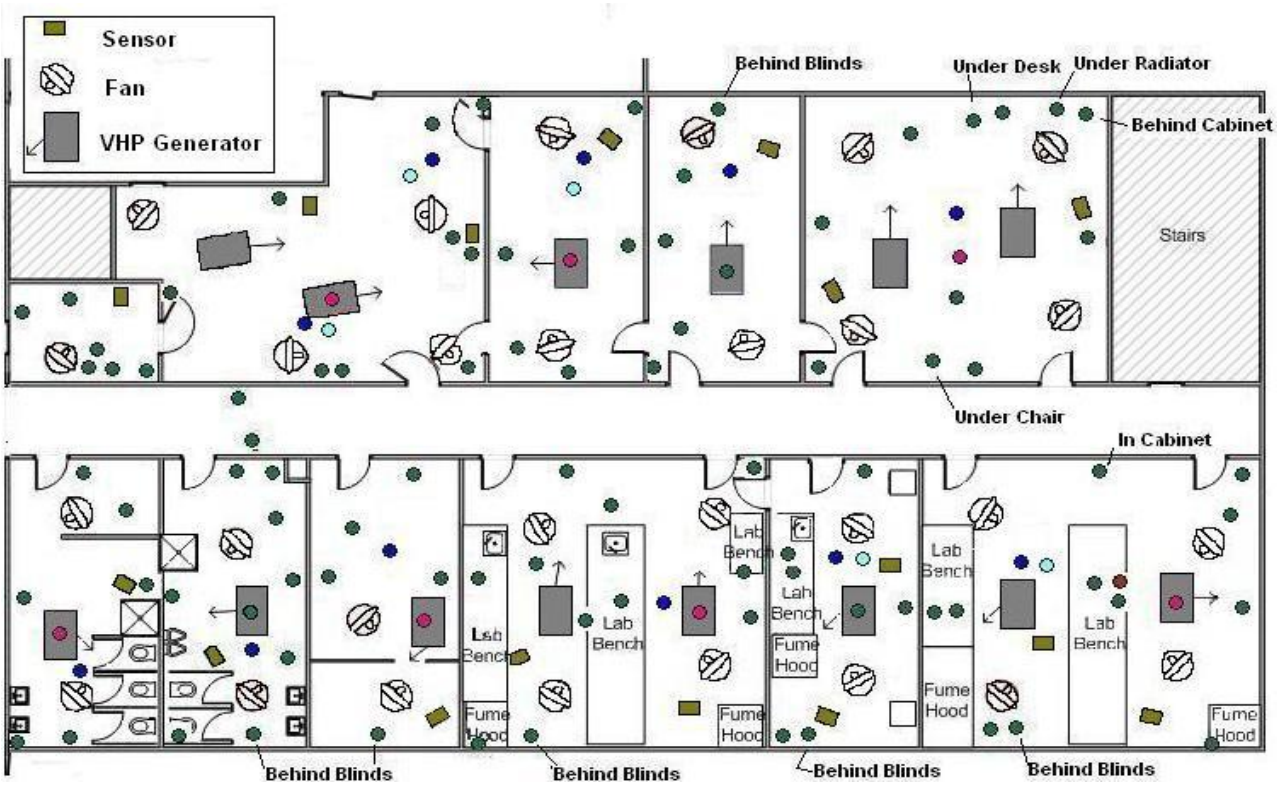

Fig. 2, Distribution of biological indicators in building-scale venue. ● wall placement; ● floor placement; ● ceiling placement, sheathed; ● ceiling placement, unsheathed

3. RESULTS

1. SUSCEPTABILITY OF *BACILLUS SPP*. TO THE VHP ANTIMICROBIAL

Bench-scale tests using four spore-forming species inoculated onto glass coupons indicated that the required exposure time to affect a 6-log reduction appeared to be species-dependent (Fig. 3). A greater than 6-log reduction in the number of viable spores was achieved *for B. anthracis, B. atrophaeus* and *B. thuringiensis* in 10, 15 and 30 minutes, respectively.

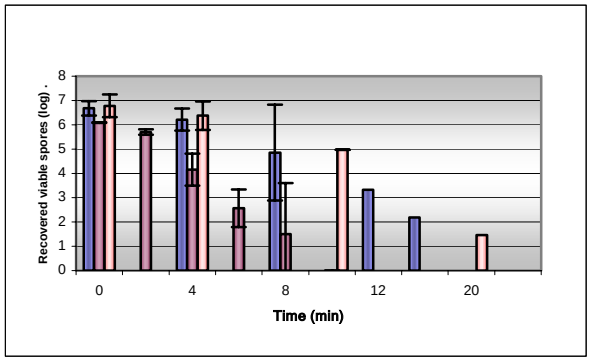

Fig. 3. *Bacillus spp*. survival during VHP exposure. ■ *B. atrophaeus,* ■ *B. anthracis,* ■ *B. thuringiensis*

2. SUSCEPTIBILITY OF *Y. RUCKERI* TO THE VHP ANTIMICROBIAL

Preliminary efforts to sustain the viability of *Y. ruckeri* upon drying were based upon protocols described by Rose, *et al* (Rose, L. J. *et al.*, 2003) in which maintenance conditions included the use of a humidity chamber.

Recovery of dried *Yersinia* cells from coupons maintained within the confines of a sterile, humidity saturated chamber retained viability for greater than 2 minutes. Equivalent cell preparations placed upon glass coupons and exposed to the VHP antimicrobial demonstrated that a greater than 6-log reduction in the number of viable cells could be achieved by a 90 sec period of exposure (Fig. 4).

3. EFFECT OF ADJUVANT ON RATE OF DECONTAMINATION

The addition of ammonia to the VHP antimicrobial vapor stream impacted slightly the exposure time required to achieve a 6-log reduction in viable Bacillus spores. Preliminary experiments in

which bacterial spores were dried upon glass coupons demonstrated that the exposure time required to achieve a 6-log reduction in viable spores was extended by approximately 25%, relative to exposure to the VHP antimicrobial alone (Fig. 6).

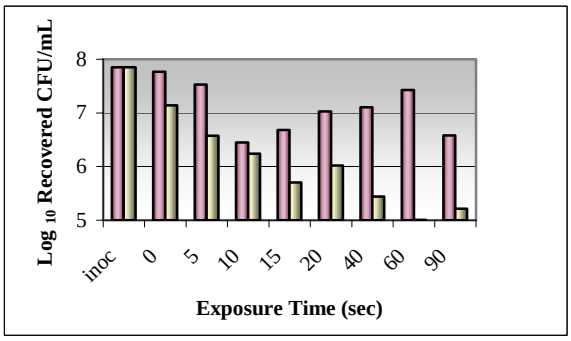

Fig. 4. Susceptibility of *Y. ruckeri* to the VHP antimicrobial unexposed cells; exposed cells

4. INFLUENCE OF SUBSTRATE ON RATE OF DECONTAMINATION

When comparing the influence of various materials regarding the efficiency of decontamination of the VHP antimicrobial, the time required for a 6-log reduction in viable cells varied (Fig. 6). The times required to reduce a $1E^7$ population of *B. anthracis* NNR1Δ1 spores dried onto either glass, aluminum, stainless steel, CARC, USAF Top Coat or Polyimid required 10, 10, 30, 30, 30, or 40 minutes of exposure, respectively, to yield a greater than 6-log reduction in the number of viable spores.

5. DECONTAMINATION OF LARGE SPACES USING THE VHP ANTIMICROBIAL

Using modular equipment provided by STE Enterprises, a VHP concentration of 275 – 350 ppm was generated within the building-scale experimental space and maintained for a period of 5 or more hours, as experimental parameters required.

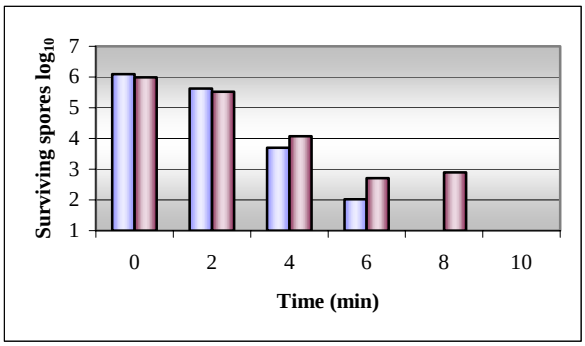

Fig. 5 Addition of Adjuvant to the VHP antimicrobial. VHP; mVHP

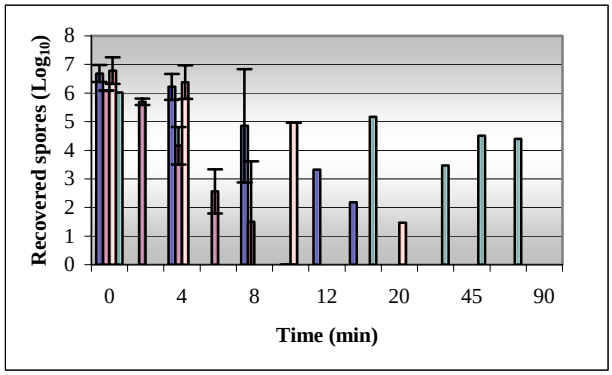

Fig. 6. Influence of substrate material on VHP efficacy – *B, anthracis* NNR1Δ1. Glass; CARC; USAF Top Coat; Aluminum; Stainless Steel; Polyimid

No variability was observed between sheathed and unsheathed coupons, analyzed in parallel.

The inoculation of sterile growth media with biological indicators following 5 hours of exposure to the VHP antimicrobial demonstrated that 99.6% (279/280) of the sheathed *G. stearothermophilus* biological indicators had been rendered non-viable.

4. DISCUSSION

1. THE VHP ANTIMICROBIAL PRODUCED A GREATER THAN 6-LOG REDUCTION OF BIOLOGICAL AGENT SURROGATES

In bench-scale testing, the exposure of 275 – 350 ppm of the VHP antimicrobial was sufficient to achieve a greater than 6-log reduction in the number of recovered viable cells. The length of time required to achieve a 6-log reduction was dependent upon two parameters: the species of test organism and the type of coupon material.

Gram-negative bacterial cells are very sensitive to desiccation (Garcia de Castro, A. *et al.*, 2000). Data depicted in Fig. 6 suggest that *Y. ruckeri* is capable of resuming normal functions when rehydrated in a humidified atmosphere.

As expected (Bender, G. R. *et al.*, 1985), *G. stearothermophilus* proved to be the most resistant, of all strains tested, to the VHP antimicrobial, when inoculated onto glass coupons, whereas *B. thuringiensis* appeared to be the most susceptible.

Coupon surface texture, porosity, chemical composition, surface cleanliness and hydrophobicity may contribute to the efficacy of the VHP antimicrobial for the decontamination of the test surface. Surface affects, depicted in Fig. 6 suggest that surface

composition can impact the design of decontamination protocols significantly.

Due to the observed surface effects, additional analysis of coupon surface characteristics is warranted. Such analysis will be informative regarding the reactivity of surface coating with the VHP antimicrobial.

REFERENCES

Bender, G. R. and R. E. Marquis, 1985: Spore heat resistance and specific mineralization. *Appl Environ Microbiol*, **50,** 1414-1421.

Dang, J. L., K. Heroux, J. Kearney, A. Arasteh, M. Gostomski, and P. A. Emanuel, 2001: Bacillus Spore Inactivation Methods Affect Detection Assays. *Appl. Environ. Microbiol.*, **67,** 3665-3670.

Garcia de Castro, A., H. Bredholt, A. R. Strom, and A. Tunnacliffe, 2000: Anhydrobiotic Engineering of Gram-Negative Bacteria. *Appl. Environ. Microbiol.*, **66,** 4142-4144.

Heckert, R., M. Best, L. T. Jordan, G. C. Dulac, D. L. Eddington, and W. G. Sterritt, 1997: Efficacy of Vaporous Hydrogen Peroxide against Exotic Animal Viruses. *Appl Environ Microbiol*, **63,** 3916-3918.

Jahnke, M. and G. Lauth, 1997: Biodecontamination of a large volume filling room with hydrogen peroxide. *Pharm. Eng.*, **17,** 2-12.

McDonnell, G., G. Gringol, and K. Antloga, 2002: Vapor phase hydrogen peroxide decontamination of food contact surfaces. *Dairy, Food Environ. Sanit.*, **22,** 868-873.

Rose, L. J., R. Donlan, S. N. Banerjee, and M. J. Arduino, 2003: Survival of *Yersinia pestis* on Environmental Surfaces. *Appl. Environ. Microbiol.*, **69,** 2166-2171.